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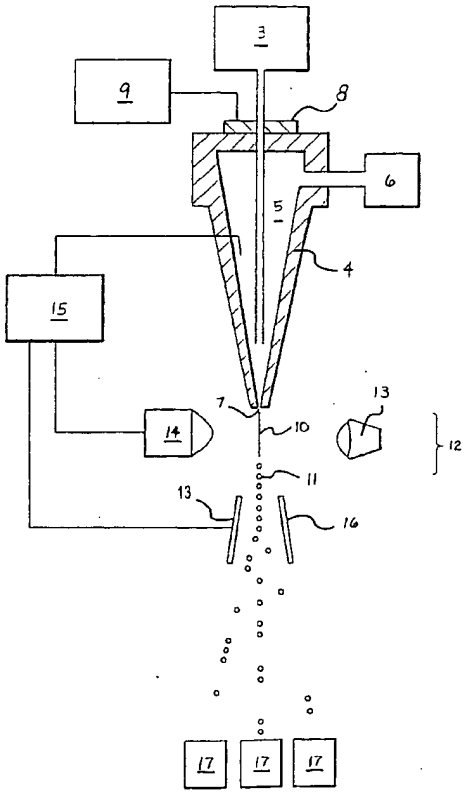

(57) Abstract: Devices, compositions, and methods for handling, separating, packaging, and utilization of spermatozoa (1) that can be derived from previously frozen sperm sampels collected forma male mammel.Specifically, techniques to uniformily stain (2) spermatozoal DNA even when derived from previously frozen sperm and separation techniques to separate and isolate spermatozoa even when derived from previously frozen sperm samples into X-chromosome bearing and Y-chromosome bearing populations having high purity.

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SYSTEM TO SEPARATE FROZEN-THAWED SPERMATOZOA INTO X-CHROMOSOME BEARING AND Y-CHROMOSOME BEARING POPULATIONS

This application claims the benefit of United States Provisional Patent Application No. 60/253,787, filed November 29, 2000 and United States Provisional Patent Application No. 60/253,785, filed November 29, 2000, each hereby incorporated by reference herein.

I. TECHNICAL FEILD

The invention involves the substantially uniform binding of fluorochrome(s) to the DNA within mammalian spermatozoa (or sperm cells) allowing such labeled spermatozoa to be separated into high purity X-chromosome bearing and Y-chromosome bearing populations. Specifically, methods for the substantially uniform binding of fluorochrome(s) to the DNA of mammalian spermatozoa contained within previously frozen and then thawed semen. In addition, the invention further involves devices, methods, and compositions for the use of high purity separated X-chromosome bearing and Y-chromosome bearing populations of spermatozoa from previously frozen-thawed semen in processes involving, but not limited to, artificial insemination, surgical insemination, and in-vitro fertilization and embryo culturing techniques.

II. BACKGROUND

Sperm can be collected from a great variety of mammals and then separated into X-chromosome bearing and Y-chromosome bearing populations based upon the difference in DNA content. In some conventional methods of spermatozoa separation, the DNA content of the spermatozoa to be separated can be stained with a fluorochrome(s) that upon excitation emit(s) a measurable amount of fluorescence. Because X-chromosome bearing spermatozoa contain a greater amount of DNA than Y-chromosome bearing spermatozoa, each X-chromosome bearing spermatozoa has the capacity to bind a relatively greater amount of fluorochrome than the corresponding Y-chromosome bearing spermatozoa. Comparison of the relative magnitude of emitted fluorescence upon excitation of the fluorochrome(s) allows the isolation of X-chromosome bearing spermatozoa from Y-chromosome bearing spermatozoa as described by United States Patent No. 5,135, 759, hereby incorporated by reference.

Even though X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa have been differentiated by and separated based upon the difference in emitted fluorescence for many years, and even though there is large commercial market for isolated populations of X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa, there remain significant problems yet to be resolved.

A significant problem with conventional methods of separating X-chromosome bearing spermatozoa from Y-chromosome bearing spermatozoa can be that each resulting population contains a significant number of incorrectly separated spermatozoa that belong in the other population. This problem in differentiating between spermatozoa can, in part, be attributed to the lack of uniformity in the amount of fluorochrome bound to the spermatozoal DNA. As such, a range in the amount of fluorochrome bound by X-chromosome bearing spermatozoa is generated and a range in the amount of fluorochrome bound by Y-chromosome bearing spermatozoa is generated. When these ranges in the amount of fluorochrome overlap or yield some values that are similar, it can be difficult or impossible to classify those individual spermatozoa to one population or the other with any degree of certainty and cross contamination of the populations can occur.

This particular problem can be exacerbated with regard to spermatozoa obtained from frozen and subsequently thawed mammalian semen. The mean purity for separatedY-chromosome bearing spermatozoa population derived from previously frozen-thawed semen can be 85% or less, and the mean purity for separated X-chromosome bearing spermatozoa population derived from previously frozen-thawed semen can be 82% or less.

Another significant problem associated with staining of spermatozoal DNA can be the detrimental effects on fertilization rates and subsequent embryonic development of fertilized oocyte(s) (oocyte, ootid, or ovum, or a plurality of same, as may be appropriate within a specific application). One aspect of this problem may be that the amount of stain bound to the DNA may effect the viability of the spermatozoa resulting in lower fertilization rates. Another aspect of this problem can be that the amount of time that elapses during the staining of the DNA may effect the viability of the sperm resulting in lower fertilization rates.

Another aspect of this problem may be that the amount of time that elapses during staining of the DNA may lower subsequent cleavage rates of oocytes fertilized with such stained spermatozoa. A 20% decline in cleavage rates have been observed for oocytes when staining time requires 190 minutes as compared to when staining time requires 60 minutes. Another aspect of this problem may be that the percent of oocytes fertilized with stained spermatozoa that proceed to blastulation may be lower as described in the journal article entitled "In vitro Fertilization with Flow-Cytometrically-Sorted Bovine Sperm", Theriogenology 52: 1393-1405 (1999), hereby incorporated by reference herein.

Another significant problem may be that cryopreserved sperm may demostrate increased capacitation, and the length of time such spermatozoa are viable may be shortened. As such, if previously frozen spermatozoa are to be separated into X-chromosome bearing and Y-chromosome bearing populations that are to be subsequently used in applications such as in-vitro fertilization, in-vivo artificial insemination, or the like, then routine staining procedures may have to be abbreviated to maintain suitable number of viable sperm cells.

As relating to the problems of staining spermatozoa uniformly, even when spermatozoa are obtained from previously frozen-thawed semen; maintaining sperm viability; separating stained spermatozoa into X-chromosome bearing and Y-chromosome bearing populations, even when the spermatozoa being separated are obtained from previously frozen semen; generating populations of X-chromosome bearing and Y-chromosome bearing spermatozoa having high purity; and successfully using separated spermatozoa for artificial insemination, surgical insemination, and in-vitro fertilization techniques it can be understood there are significant problems with conventional technology which are addressed by the instant invention.

III. DISCLOSURE OF THE INVENTION

A broad object of embodiments of the invention can be to provide DNA staining technology that allows substantially uniform amounts of fluorochrome to be bound to the DNA of all individual spermatozoa bearing an X-chromosome and substantially uniform

amounts of fluorochrome to be bound to all individual spermatozoa bearing a Y-chromosome within an amount of semen.

One aspect of this broad object of the invention can be to narrow the range in magnitude of emitted fluorescence for each of the X-chromosome bearing population and the Y-chromosome bearing population of spermatozoa upon passing through a fluorochrome excitation source.

Another aspect of this broad object of the invention can be to increase the difference between the mean values of magnitude of emitted fluorescence for each of the X-chromosome bearing population and the Y-chromosome bearing population of spermatozoa upon passing through a fluorochrome excitation source.

Another aspect of this broad object of the invention can be to decrease the number of spermatozoa incorrectly assigned to each of the X-chromosome bearing population and the Y-chromosome bearing population of spermatozoa.

Another aspect of this broad object of the invention can be to generate separate X-chromosome bearing and Y-chromosome bearing populations having greater than 85% purity or greater than 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or even 99% purity.

Another broad object of embodiment of the invention can be to allow assessment of a wide range of genetics. Rather than being limited to the genetics of individuals from species of mammals having proximity to a spermatozoa separating or sorting facility, genetics representing a wide variety of individuals from numerous species can be transported as frozen semen to distant spermatozoa separation facilities for subsequent separation into X-chromosome bearing and into Y-chromosome bearing populations. These species of mammals may include, but are not limited to primates, such as chimpanzees, gorillas, humans, or the like; marine mammals, such as whales, porpoises, or the like; bovids; ovids; swine; canids; felids; or equids, as but a few examples. It may also include genetics that are

considered rare because the species of mammal may be endangered or few in number; or considered rare because the individual has desirable morphological, physiological, or intellectual attributes.

Another broad object of embodiments of the invention can provide separation technology for differentiating between X-chromosome bearing and Y-chromosome bearing spermatozoa obtained from frozen-thawed semen.

Another object of embodiments of the invention can be to provide DNA staining technology to more uniformly stain the DNA of spermatozoa contained in frozen-thawed semen to improve the apparent resolution between X-chromosome bearing and Y-chromosome bearing spermatozoa.

Another object of embodiments of the invention can be to provide high purity artificial insemination samples prepared from separated spermatozoa from frozen-thawed semen.

Another object of embodiments of the invention can be to provide high purity low dose artificial insemination samples prepared from separated spermatozoa from frozen-thawed semen.

Another object of embodiments of the invention can be to provide high purity insemination samples for surgical insemination procedures prepared from separated spermatozoa from frozen-thawed semen.

Another object of an embodiment of the invention can be to provide high purity insemination samples for in-vitro fertilization procedures prepared from separated spermatozoa from frozen-thawed semen.

Another object of an embodiment of the invention can be to provide high purity insemination samples for in-vitro fertilization procedures prepared from separated

spermatozoa from frozen-thawed human semen.

Another object of an embodiment of the invention can be to provide technology for staining and separation of spermatozoa from frozen-thawed sperm into X-chromosome bearing populations and Y-chromosome bearing populations for in-vitro fertilization of oocyte(s) that is not detrimental to cleavage rates or embryonic development.

Naturally further objects of the invention are disclosed throughout other areas of specification.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a particular embodiment of the invention for staining the DNA of spermatozoa contained in frozen-thawed semen.

Figure 2 shows a particular embodiment of the invention for separating spermatozoa from frozen-thawed semen into X-chromosome bearing and Y-chromosome bearing spermatozoa.

Figure 3 shows a further view of a particular embodiment of the invention for separating spermatozoa from frozen-thawed semen into X-chromosome bearing and Y-chromosome bearing spermatozoa.

V. MODE(S) FOR CARRYING OUT THE INVENTION

To routinely separate spermatozoa (live, fixed, viable, non-viable, or nuclei) into high purity X-chromosome bearing samples and into Y-chromosome bearing samples, the method used to sort the X-chromosome bearing and Y-chromosome bearing spermatozoa must provide sufficient resolution of the X-chromosome bearing spermatozoa from the Y-chromosome bearing spermatozoa so that separation or sorting step(s) can be achieved without substantial cross contamination.

Resolution or differentiation of spermatozoa can be based upon ascertaining the

difference in the fluorescent emission from the amount of fluorochrome bound to the DNA within the X-chromosome bearing spermatozoa upon excitation and the fluorescent emission from the amount of fluorochrome bound to the DNA within the Y-chromosome bearing spermatozoa upon excitation. Separation of X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa based upon this measurable difference may then be achieved by a number of methods such as flow cytometry, liquid chromatography, gel electrophoresis, and other technologies that similarly compare the relative magnitude of fluorescence to differentiate between X-chromosome bearing spermatozoa and the Y-chromosome bearing spermatozoa.

Spermatozoa separation systems can have problems differentiating between the fluorescent emission generated by the fluorochrome bound to the DNA of X-spermatozoa, and the fluorescent emission generated by the fluorochrome bound to the DNA of Y-spermatozoa upon excitation when the amount of the fluorochrome bound to the DNA of individual spermatozoa is not consistent within the Y-chromosome bearing or X-chromosome bearing populations. These difficulties in differentiating between the amount of fluorescent emissions generated by the bound fluorochrome(s) become exacerbated when spermatozoa are obtained from frozen-thawed sperm which are stained by conventional techniques.

The failure to stain the spermatozoal DNA consistently can generate a broader range of fluorescing species for both X-chromosome bearing and Y-chromosome bearing populations of spermatozoa. This broader range of fluorescing species for the two populations results in an increased range of apparent DNA molecular weights and a decreased ability to resolve X-chromosome bearing from Y-chromosome bearing spermatozoa. The decrease in resolution makes separation of the X-chromosome bearing spermatozoa from the Y-chromosome bearing spermatozoa more difficult and results in cross contamination between populations and a lower purity of separated spermatozoa samples are obtained.

Particular embodiments of the invention provide technology to stain the DNA of live

viable spermatozoa or the spermatozoal DNA of frozen-thawed semen specimens to allow increased resolution of X-chromosome bearing from the Y-chromosome bearing spermatozoa resulting in high purity X-chromosome bearing and high purity Y-chromosome bearing populations of sperm cells. As such, it is understood that the term high purity can mean greater resolution of the X-chromosome bearing from the Y-chromosome bearing spermatozoa compared to conventional staining technology for a given application. High purity can also mean less cross contamination between separated spermatozoa populations compared to conventional separation technologies.

For example, in particular flow cytometry embodiments of the invention, high purity for stained frozen-thawed live spermatozoa can mean sorted populations of X-chromosome bearing spermatozoa and Y-chromosome bearing spermatozoa having a purity greater than about 85%. However, if live viable sperm or sperm nuclei are being sorted high purity may mean X-chromosome bearing and Y-chromosome bearing populations having a purity greater than about 90%. As can be understood, the definition of high purity is contextual involving a comparison of the results obtained from each embodiment of the invention compared to the results obtained when utilizing convention technologies for a particular application. In the context of spermatozoa having DNA that stains poorly, such as previously frozen-thawed spermatozoal DNA, high purity can mean populations of isolated spermatozoa bearing greater than 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% of either an X-chromosome or a Y-chromosome.

Embodiments of the invention can include spermatozoa collected from numerous species of male mammals, and the invention should be understood not to be limited to the species of male mammals described by the specific examples within this application. Rather the specific examples within this application are intended to be illustrative of the varied and numerous species of male mammals from which semen can be collected and utilized in certain embodiments of the invention. Embodiments of the invention, for example, may include the spermatozoa of animals having commercial value for meat or dairy production such as swine, ovids, bovids, equids, buffalo, or the like (naturally the mammals used for meat or dairy production may vary from culture to culture). It may also include the

spermatozoa of various domesticated mammalian species encompassed by canids and felids. It may also include spermatozoa from individuals of various mammalian species that have uncommon attribute(s), such as morphological characteristics including weight, size, or conformation, or other desired characteristics such as speed, agility, intellect, or the like. It may also include spermatozoa of primates, including but not limited to chimpanzees, gorillas, or humans and the spermatozoa from marine mammals such as whales and dolphins. It may also include frozen-thawed spermatozoa from all the various mammals above-described and further, including but not limited to, the spermatozoa of deceased donors, from rare or exotic mammals, zoological specimens, or endangered species.

Now referring primarily to Figure 1, particular embodiments of the invention can comprise semen containing spermatozoa (1) collected from a male mammal, including but not limited to, those above-described. The spermatozoa can be incubated in a concentration of Hoechst 33342 stain (2) of greater than about 40 μM at a temperature between about 30 ° Centigrade and about 40° Centigrade for a duration of time between 50 minutes to 200 minutes to stain spermatozoal DNA with sufficient uniformity to allow X-chromosome bearing spermatozoa to be differentiated from Y-chromosome bearing spermatozoa based upon the magnitude of fluorescence at a rate greater than about 85%.

The concentration of Hoechst 33342 stain between 40 μM and 2500 μM , the temperature between 30 ° Centigrade and about 40° Centigrade, and the duration of time between 50 minutes and 200 minutes can be selected to adjust the purity of the separated X-chromosome bearing and Y-chromosome bearing populations, or can be selected to promote cleavage rates and embryonic development, as further discussed below.

For example, when staining spermatozoal DNA from certain bovine species, the concentration of Hoechst 33342 can be increased to between about 200 μM and about 2500μM, incubated for a period of time between about 60 minutes to about 190 minutes at a temperature of about 37 ° Centigrade. Specifically with respect to certain frozen-thawed bovine spermatozoa, the Hoechst 33342 stain (2) can be adjusted to establish a concentration of 2240μM and then incubated for about 60 minutes at about 39 ° Centigrade.

With respect to the cleavage rates of oocytes inseminated with mammalian sperm cells treated according to the invention, the increase in stain concentration up to at least 2240μM does not appear to have a depressive effect on either cleavage or embryonic development. Higher stain concentrations may actually be beneficial with respect to certain embodiments of the invention because the length of incubation time may be decreased improving percent cleavage or blastocyst formation. From application to application the concentration of Hoechst 33342, the length of incubation time, or both can be adjusted to obtain the maximal cleavage rate and blastocyst formation, if desired.

Now referring primarily to Figures 2 and 3, flow cytometric embodiments of the invention can include a cell source (3) which acts to establish or supply stained spermatozoa (fresh, frozen-thawed, sperm nuclei, or the like) to be analyzed by flow cytometry. The cells are deposited within a nozzle (4) in a manner such that the stained sperm cells are surrounded by a sheath fluid (5). The sheath fluid (5) is usually supplied by a sheath fluid source (6) so that as the cell source (3) supplies sperm cells, the sheath fluid (5) is concurrently fed through the nozzle (4). In this manner the sheath fluid (5) forms a sheath fluid environment for the sperm cells. Since the various fluids are provided to the flow cytometer at some pressure, they flow out of the nozzle (4) and exit at the nozzle orifice (7). By providing a type of oscillator (8) which may be very precisely controlled through an oscillator control (9), pressure waves may be established within the nozzle (4) and transmitted to the fluids exiting the nozzle (4) at the nozzle orifice (7). Since the oscillator (9) acts upon the sheath fluid (5), the stream (10) exiting the nozzle orifice (7) eventually and regularly forms drops (11). Because the sperm cells are at least partially surrounded by a sheath fluid environment, the drops (11) can contain within them individually isolated sperm cells.

Since the drops (11) generally contain individual isolated sperm cells, the flow cytometer can distinguish and separate droplets based upon the magnitude of fluorescence emitted from the fluorochrome bound to the spermatozoal DNA. This is accomplished through a cell sensing system (12). The cell sensing system involves at least some type of sensor (13) which responds to the magnitude of fluorescence emitted by each sperm cell contained within each drop (11). The sperm cell sensing system (13) may cause an action

depending upon the relative presence or relative absence of fluorescence emitted by the bound fluorochrome upon excitation by some stimulant such as the laser exciter (14). While each spermatozoon can be stained by the fluorochrome, such as Hoechst 33342, as described above, the differing amount of DNA comprising the X-chromosome and the Y-chromosome causes different amounts of stain to be bound. Thus, by sensing the degree of fluorescence emitted by the fluorochrome upon excitation it is possible to discriminate between X-bearing spermatozoa and Y-bearing spermatozoa by their differing emission levels.

In order to achieve separation and isolation of the appropriate sperm cells, the signals received by sensor (14) are fed to some type of sorter discrimination system (15) which very rapidly makes a differentiation decision and can differentially charge each drop (11) based upon whether it has decided that the desired sperm cell does or does not exist within that drop (11). In this manner the separation or discrimination system (15) acts to permit the electrostatic deflection plates (16) to deflect drops (11) based on whether or not they contain the appropriate sperm cell. As a result, the flow cytometer acts to sort cells by causing them to land in one or more collectors or containment elements (17). Thus by sensing some property of the sperm cells (such as magnitude of fluorescense), the flow cytometer can discriminate between sperm cells based on that particular characteristic and place them in the appropriate collector or containment element (17). In particular embodiments of the invention using flow cytometry to sort spermatozoa, the X-bearing sperm cell containing droplets are charged positively and thus deflect in one direction, and the Y-bearing sperm cell containing droplets are charged negatively and thus deflect the other way, and the wasted stream (containing unsortable sperm cells) remain uncharged and thus can be collected in an undeflected stream into a suction tube, or the like.

Now referring primarily to Figure 3, the nozzle (4) emits a stream (10) which because of the oscillator (8) (not shown in Figure 3) forms drops (11). Since the sperm cell source (3) (not shown in Figure 3) may supply sperm cells (1) which may be stained according to the above-described invention, the light emission from the bound fluorochrome excited by laser exciter (13) can be differentially determined by sensor (14) so that the existence or nonexistence of a charge on each drop (11) as it separates from stream (10) can be controlled

by the flow cytometer. This control results in positively charged, negatively charged, or uncharged drops (8) based upon the sperm cell contained within each drop (11). As shown by Figure 3, certain drops are shown as deflected drops (18). These deflected drops (18) are those containing spermatozoon differentiated by bearing either an X-chromosome or a Y-chromosome. Separated spermatozoa are then isolated in an appropriate collection element or containment element (17) for later use.

Embodiments of the invention can comprise droplets (11) each containing a sperm cell (15) bearing either an X-chromosome or a Y-chromosome. Droplets containing X-chromosome bearing sperm cells can be isolated into containment element(s) (17) at a rate of at least 1000 per second or at a rate greater than about 1000 per second, such as 2000 per second, 3000 per second, 4000 per second, 5000 per second, or higher. Similarly Y-chromosome bearing sperm cells can be isolated at a rate of at least 1000 per second or at a rate greater than about 1000 per second, such as 2000 per second, 3000 per second, 4000 per second, 5000 per second, or higher. In some embodiments of the invention, droplets containing X-chromosome bearing sperm cells and droplets containing Y-chromosome bearing sperm cells are simultaneously separated and isolated into containment elements each at a rate of at least 1000 per second, or greater than 1000 per second, such as 2000 per second, 3000 per second, 4000 per second, 5000 per second, or at even higher rates.

Embodiments of the invention can also include artificial insemination samples prepared from sperm cells collected from male mammals (which can be frozen and thawed with respect to some embodiments of the invention) that are then stained and separated according to embodiments of the invention above-described. The artificial insemination samples can then be utilization in artificial insemination protocols. For example, a bovine artificial insemination sample prepared from separated spermatozoa according to the invention can comprise fewer than 10×10^6 viable spermatozoa contained within a straw. Low dose artificial insemination samples for bovine artificial insemination can contain as few as $1\text{-}3 \times 10^6$ viable spermatozoa, or even as few as 150,000 spermatozoa as described in United States Patent Application 09/001,394, or PCT Patent Application US98/27909, each hereby incorporated by reference. Artificial insemination samples, having a regular number

of separated sperm cells or a low dose of separated sperm cells can be used in animal breeding programs, such as those described in United States Patent Applications 60/224,050 and 60/21,093, each hereby incorporated by reference. Artificial insemination samples containing previously frozen and thawed spermatozoa stained and separated according to the invention can also be utilized in conjunction with synchronized breeding programs using superovulated animals as described in United States patent Application 09/001,454, hereby incorporated by reference herein. Naturally, for frozen sperm cells that are of limited availability because the male mammal is deceased, or the male mammal is a rare or exotic animal, an artificial insemination sample prepared according to the invention may contain even fewer spermatozoa.

The number of viable separated spermatozoa that are stained, separated, and isolated into X-chromosome bearing or Y-chromosome bearing populations according to the invention that are used in an artificial insemination sample can vary based upon the species of mammal to be artificially inseminated. For example, equine artificial insemination samples prepared from separated spermatozoa may require a higher number of viable separated spermatozoa relative to the bovine application, as described in PCT Patent Application US99/17165, hereby incorporated by reference. An embodiment of an equine insemination sample may, as but one example, contain between about forty million to about one-hundred million spermatozoa.

In certain embodiments of the invention, the insemination sample containing separated spermatozoa collected from a male mammal or obtained from frozen-thawed sperm may be packaged for use with surgical insemination procedures

Sperm cells stained, separated, or isolated according to the invention can also be used to fertilize oocyte(s) in-vitro (IVF). An attractive feature of IVF can be that fewer separated sperm are need than for artificial insemination. It may be desirable to use the fewest sperm possible, especially if the male mammal is deceased, rare, or exotic or if the spermatozoa are stained or separated in accordance with various embodiments of the invention. Also, commercial availability of sperm cells separated into X-chromosome bearing and Y-

chromosome bearing populations, especially when the male mammal is located a distance from the female mammal, or is exotic, rare, or has desirable attributes, will likely result in greatly expanded use of IVF in breeding programs. Certain embodiments of the invention can include devices and methodologies for the use of separated spermatozoa, including but not limited to frozen-thawed sperm cells, with respect to the in-vitro fertilization of oocytes, the in-vitro oocyte maturation, or the in-vitro culture of zygotes, such as those described in the journal article by Lu, K.H., Cran D.G., and Seidel, G. E., In-vitro Fertilization With Flow Cytometrically-Sorted Bovine Sperm, Theriogenology, 52, 1393-1405 (1999), hereby incorporated by reference.

Certain embodiments of the invention involving the production or generation of mammalian embryos can comprise collection of semen (1) from a male mammal or obtaining semen or spermatozoa (1) that are or have been previously frozen. According to embodiments of the invention described above, the semen is combined wtih Hoechst 33342 (2) stain to establish a concentration of between 40 μM and 2500 μM. The sperm cells are incubated with the Hoechst 33342 stain at a temperature between about 30 ° Centigrade and about 40° Centigrade for a duration of between about 50 minutes to about 200 minutes. The stained sperm cells may be separated and isolated into X-chromosome bearing and Y-chromosome bearing populations according to embodiments of the invention described above or by other sperm cell separation techniques that also differentiate X-chromosome bearing spermatozoa from Y-chromosome bearing spermatozoa based upon the magnitude of fluorescence. The isolated sperm cells may then be used to fertilize oocytes from a female mammal of the same species, and in some cases from female mammals of different species, in-vitro.

As an example of an application of embodiments of the invention involving frozen bull sperm in IVF applications, sperm samples from two bulls were stained either at a concentration of 224 μM or 2,240 μM of Hoechst 33342 and the stained spermatozoa were then bulk sorted on a flow cytometer at 1000 sperm/sec into 2% egg yolk citrate. Spermatozoa were inseminated at $1x10^6$/mL and embryos were cultured in the mSOF system described by Tervit H.R. et al., Successful Culture In-Vitro of Sheep and Cattle Ova, J.

Reprod. Fertil., 30:493-497 (1992), hereby incorporated by reference. Three replicates were carried out for bull 1 and one replicate for bull 2 (Table 1).

Table 1. Effect of stain concentration on cleavage and developmental rates of oocytes inseminated with separated stained spermatozoa from frozen-thawed sperm.

Bull	No. Ejaculates	Hoechst 33342 conc. (μM)	Staining time required (min)	No. oocytes	% cleave	% blastocysts/ oocyte
1	3	224	190	368	44[a]	17
1	3	2240	60	373	60[b]	23
2	1	224	190	86	23[a]	0[a]
2	1	2240	60	81	42[b]	16[b]

[a,b] Percentages within bulls within columns with different superscripts differ ($P<.025$, χ^2)

As can be understood , It can take much longer to stain frozen-thawed sperm so that they can be resolved during separation at the lower stain concentration than at 10X stain concentration. The differences observed in cleavage rates between the two stain concentrations most likely can be attributed to the extended incubation time at the lower stain level. It appears that a 10-fold increase in stain concentration does not have depressive effect on either cleavage of embryonic development.

As can be easily understood from the foregoing, the basic concepts of the present invention may be embodied in a variety of ways. It involves the staining of spermatozoa, whether fresh spermatozoa or frozen-thawed spermatozoa, separation and isolation techniques which may be used with such stained spermatozoa, as well as devices to accomplish the staining, separation, and isolation of such stained spermatozoa into X-chromosome bearing and Y-chromosome bearing populations. In this patent application, the staining and separating techniques used with spermatozoa are disclosed as part of the results

shown to be achieved by the various devices described and as steps which are inherent to utilization. They are simply the natural result of utilizing the devices as intended and described. In addition, while some devices are disclosed, it should be understood that these not only accomplish certain methods but also can be varied in a number of ways. Importantly, as to all of the foregoing, all of these facets should be understood to be encompassed by this disclosure.

The discussion included in this international Patent Cooperation Treaty patent application is intended to serve as a basic description. The reader should be aware that the specific discussion may not explicitly describe all embodiments possible; many alternatives are implicit. It also may not fully explain the generic nature of the invention and may not explicitly show how each feature or element can actually be representative of a broader function or of a great variety of alternative or equivalent elements. Again, these are implicitly included in this disclosure. Where the invention is described in functionally-oriented terminology, each aspect of the function is accomplished by a device, subroutine, or program. Apparatus claims may not only be included for the devices described, but also method or process claims may be included to address the functions the invention and each element performs. Neither the description nor the terminology is intended to limit the scope of the claims which now be included.

Further, each of the various elements of the invention and claims may also be achieved in a variety of manners. This disclosure should be understood to encompass each such variation, be it a variation of an embodiment of any apparatus embodiment, a method or process embodiment, or even merely a variation of any element of these. Particularly, it should be understood that as the disclosure relates to elements of the invention, the words for each element may be expressed by equivalent apparatus terms or method terms -- even if only the function or result is the same. Such equivalent, broader, or even more generic terms should be considered to be encompassed in the description of each element or action. Such terms can be substituted where desired to make explicit the implicitly broad coverage to which this invention is entitled. As but one example, it should be understood that all actions may be expressed as a means for taking that action or as an element which causes that action.

Similarly, each physical element disclosed should be understood to encompass a disclosure of the action which that physical element facilitates. Regarding this last aspect, as but one example, the disclosure of a "sorter" should be understood to encompass disclosure of the act of "sorting" -- whether explicitly discussed or not -- and, conversely, were there only disclosure of the act of "sorting", such a disclosure should be understood to encompass disclosure of a "sorter" and even a "means for sorting". Such changes and alternative terms are to be understood to be explicitly included in the description. Additionally, the various combinations and permutations of all elements or applications can be created and presented. All can be done to optimize the design or performance in a specific application.

Any acts of law, statutes, regulations, or rules mentioned in this application for patent: or patents, publications, or other references mentioned in this application for patent are hereby incorporated by reference. Specifically, United States Provisional Patent Application No. 60/253,787, filed November 29, 2000 and United States Provisional Patent Application No. 60/253,785, filed November 29, 2000, are hereby incorporated by reference including any figures or attachments, and each of references in the following table of references are hereby incorporated by reference.

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In addition, as to each term used it should be understood that unless its utilization in this application is inconsistent with such interpretation, common dictionary definitions should be understood as incorporated for each term and all definitions, alternative terms, and synonyms such as contained in the Random House Webster's Unabridged Dictionary, second edition are hereby incorporated by reference. However, as to each of the above, to the extent that such information or statements incorporated by reference might be considered inconsistent with the patenting of this/these invention(s) such statements are expressly not to be considered as made by the applicant(s).

In addition, unless the context requires otherwise, it should be understood that the term "comprise" or variations such as "comprises" or "comprising", are intended to imply the inclusion of a stated element or step or group of elements or steps but not the exclusion of any other element or step or group of elements or steps. Such terms should be interpreted in their most expansive form so as to afford the applicant the broadest coverage legally permissible in countries such as Australia and the like.

Thus, the applicant(s) should be understood to have support to claim at least: i) each of the staining, separation, isolation, insemination, or fertilization procedures as herein disclosed and described, ii) the related methods disclosed and described, iii) similar, equivalent, and even implicit variations of each of these devices and methods, iv) those

alternative designs which accomplish each of the functions shown as are disclosed and described, v) those alternative designs and methods which accomplish each of the functions shown as are implicit to accomplish that which is disclosed and described, vi) each feature, component, and step shown as separate and independent inventions, vii) the applications enhanced by the various systems or components disclosed, viii) the resulting products produced by such systems or components, ix) methods and apparatuses substantially as described hereinbefore and with reference to any of the accompanying examples, and x) the various combinations and permutations of each of the elements disclosed.

The claims set forth in this specification are hereby incorporated by reference as part of this description of the invention, and the applicant expressly reserves the right to use all of or a portion of such incorporated content of such claims as additional description to support any of or all of the claims or any element or component thereof, and the applicant further expressly reserves the right to move any portion of or all of the incorporated content of such claims or any element or component thereof from the description into the claims or vice-versa as necessary to define the subject matter for which protection is sought by this application or by any subsequent continuation, division, or continuation-in-part application thereof, or to obtain any benefit of, reduction in fees pursuant to, or to comply with the patent laws, rules, or regulations of any country or treaty, and such content incorporated by reference shall survive during the entire pendency of this application including any subsequent continuation, division, or continuation-in-part application thereof or any reissue or extension thereon.

VI. CLAIMS

I claim:

1. A method of staining sperm cells collected from mammals, comprising the steps of:
   a. collecting semen from a male mammal;
   b. incubating sperm cells contained within said semen in a concentration of Hoechst 33342 stain of greater than 40 micro-molar;
   c. establishing the temperature at which said sperm cells in said concentration of Hoechst 33342 stain are incubated between about 30 degrees centigrade and about 40 degrees centigrade;
   d. adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes; and
   e. staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85%.

2. A method of staining sperm cells collected from mammals as described in claim 1, wherein said male mammal is selected from the group of mammals consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.

3. A method of staining sperm cells collected from mammals as described in claim 2, wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.

4. A method of staining sperm cells collected from mammals as described in claim 2, wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is 224 micro-molar.

5. A method of staining sperm cells collected from mammals as described in claim 2, wherein said male mammal comprises said bovid and said concentration of Hoechst 3342 stain is 2240 micro-molar.

6. A method of staining sperm cells collected from mammals as described in claim 4, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 190 minutes.

7. A method of staining sperm cells collected from mammals as described in claim 5, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.

8. A method of staining sperm cells collected from mammals as described in claim 1, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said Y-chromosome bearing sperm cells from said X-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

9. A method of staining sperm cells collected from mammals as described in claim 8, wherein said X-chromosome bearing sperm cells differentiated from said Y-chromosome bearing sperm cells comprise viable sperm cells.

10. A method of staining sperm cells collected from mammals as described in claims 1,

2, 6, 7, 8, or 9, further comprising the step of freezing said semen.

11. A method of staining sperm cells collected from mammals as described in claim 10, further comprising the step of thawing said semen.

12. A method of staining sperm cells collected from mammals as described in claim 11, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow cytometer.

13. A method of staining DNA within frozen-thawed sperm cells, comprising the steps of:

a. collecting semen containing sperm cells from a male mammal;

b. freezing said semen containing said sperm cells;

c. thawing said semen containing said sperm cells;

d. combining frozen-thawed semen with Hoechst 33342 stain;

e. establishing a concentration of said Hoechst 33342 stain between about 200 micro-molar and about 2500 micro-molar;

f. adjusting the temperature of said frozen-thawed semen in said concentration of Hoechst 33342 stain to between about 30 degrees Centigrade and about 40 degrees Centigrade; and

g. adjusting the duration of time said frozen-thawed semen in said concentration of Hoechst 33342 stain incubates to between about 50 minutes and 200 minutes, whereby DNA within sperm cells contained in said frozen-thawed are stained with sufficient uniformity to differentiate between X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells on the basis of magnitude of fluorescence.

14. A method of staining sperm cells collected from mammals as described in claim 13,

wherein said male mammal is selected from the group of mammals consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.

15. A method of staining sperm cells collected from mammals as described in claim 14, wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.

16. A method of staining sperm cells collected from mammals as described in claim 14, wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is 224 micro-molar.

17. A method of staining sperm cells collected from mammals as described in claim 14, wherein said male mammal comprises said bovid and said concentration of Hoechst 3342 stain is 2240 micro-molar.

18. A method of staining sperm cells collected from mammals as described in claim 14, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 190 minutes.

19. A method of staining sperm cells collected from mammals as described in claim 17, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.

20. A method of staining sperm cells collected from mammals as described in claims 13, 15, 16, 17, 18, 19, or 20, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of

fluorescence at a rate of greater than about 85% comprises differentiating said Y-chromosome bearing sperm cells from said X-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

21. A method of staining sperm cells collected from mammals as described in claim 20, wherein said X-chromosome bearing sperm cells differentiated from said Y-chromosome bearing sperm cells comprise viable sperm cells.

22. A method of staining sperm cells collected from mammals as described in claim 20, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow cytometer.

23. A method of generating mammalian embryos, comprising the steps of:

a. collecting semen from a male mammal;

b. combining said semen from said male mammal with an amount of Hoechst 33342 stain;

c. establishing a concentration of Hoechst 33342 stain combined with said semen to a concentration between about 40 micro-molar and about 2500 micro-molar;

d. adjusting the temperature at which said sperm cells are incubated with said Hoechst 33342 stain between about 30 degrees centigrade and about 40 degrees centigrade;

e. adjusting the duration of time said sperm cells are incubated in said concentration of Hoechst 33342 between about 60 minutes and about 200 minutes;

f. staining DNA within sperm cells contained in said semen with said Hoechst 33342 stain; and

g. fertilizing oocytes with stained sperm cells,
whereby increasing the concentration of Hoechst 33342 stain and decreasing the duration of time said sperm cells are incubated in said concentration of Hoechst 33342 stain increases the percentage of said mammalian embryos produced.

24. A method of staining sperm cells collected from mammals as described in claim 23, wherein said male mammal is selected from the group of mammals consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.

25. A method of staining sperm cells collected from mammals as described in claim 24, wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.

26. A method of staining sperm cells collected from mammals as described in claim 24, wherein said male mammal comprises said bovid and said concentration of Hoechst 33342 stain is 224 micro-molar.

27. A method of staining sperm cells collected from mammals as described in claim 24, wherein said male mammal comprises said bovid and said concentration of Hoechst 3342 stain is 2240 micro-molar.

28. A method of staining sperm cells collected from mammals as described in claim 26, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 190 minutes.

29. A method of staining sperm cells collected from mammals as described in claim 27, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200

minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.

30. A method of staining sperm cells collected from mammals as described in claims 23, 25, 26, 27, 28, 29, or 30, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said Y-chromosome bearing sperm cells from said X-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

31. A method of staining sperm cells collected from mammals as described in claim 30, wherein said X-chromosome bearing sperm cells differentiated from said Y-chromosome bearing sperm cells comprise viable sperm cells.

32. A method of staining sperm cells collected from mammals as described in claim 30, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow cytometer.

33. A method of staining sperm cells collected from mammals as described in claim 32, further comprising the step of isolating differentiated X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells into separate collection elements.

34. A method of staining sperm cells collected from mammals as described in claim 33, wherein said step of isolating differentiated X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells into separate collection elements comprises isolating Y-chromosome bearing sperm cells into a separate collection element at a

rate of about 1000 per second.

35. A method of staining sperm cells collected from mammals as described in claim 33, wherein said step of isolating differentiated X-chromosome bearing sperm cells and Y-chromosome bearing sperm cells into separate collection elements comprises isolating X-chromosome bearing sperm cells into a separate collection element at a rate of about 1000 per second.

36. A method of generating mammalian embryos as described in claims 23, further comprising the step of freezing said semen.

37. A method of generating mammalian embryos as described in claims 30, further comprising the step of freezing said semen.

38. A method of generating mammalian embryos as described in claim 36, further comprising the step of thawing said semen.

39. A method of generating mammalian embryos as described in claim 37, further comprising the step of thawing said semen.

40. A flow cytometer system for isolating desired sperm cells, comprising:

a. sperm cells obtained by thawing previously frozen semen, wherein said sperm cells are incubated with a concentration of Hoechst 33342 stain between about 200 micro-molar and about 2500 micro-molar until DNA within said sperm cells are stained with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85%;

b. a sperm cell source that supplies said sperm cells to a flow cytometer;

c. a sheath fluid source that creates a sheath fluid environment within said flow cytometer in which said sperm cells are entrained;

d. a nozzle through which said sperm cells pass while entrained in said sheath fluid environment;

e. an oscillator that acts upon said sheath fluid as it passes through said nozzle;

f. a sperm cell sensing system responsive to said sperm cells;

g. a separation discrimination system that acts to separate said sperms cells having a desired characteristic; and

h. a containment element into which said sperm cells having said desired characteristic are collected.

41. A flow cytometer system for isolating desired sperm cells as described in claim 40, wherein said sperm cells obtained by thawing previously frozen semen are obtained from male mammals selected from the group consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.

42. A flow cytometer system for isolating desired sperm cells as described in claim 41, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.

43. A flow cytometer system for isolating desired sperm cells as described in claim 41, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is about 224 micro-molar.

44. A flow cytometer system for isolating desired sperm cells as described in claim 41, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 3342 stain is 2240 micro-molar.

45. A flow cytometer system for isolating desired sperm cells as described in claim 42, further comprising a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes.

46. A flow cytometer system for isolating desired sperm cells as described in claim 43, wherein said duration of time about 190 minutes.

47. A flow cytometer system for isolating desired sperm cells as described in claim 44, wherein said duration of time about 60 minutes.

48. A flow cytometer system for isolating desired sperm cells as described in claims 40, 42, 43, 45, or 48, wherein said X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% is a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

49. A flow cytometer system for isolating desired sperm cells as described in claim 48, further comprising a collection element into which differentiated X-chromosome bearing sperm cells are isolated.

50. A flow cytometer system for isolating desired sperm cells as described in claim 48, further comprising a collection element into which differentiated Y-chromosome bearing sperm are isolated.

51. A flow cytometer system for isolating desired sperm cells as described in claim 49, further comprising a rate at which X-chromosome bearing sperm cells are isolated greater than about 1000 per second.

52. A flow cytometer system for isolating desired sperm cells as described in claim 50, further comprising a rate at which Y-chromosome bearing sperm cells are isolated greater than about 1000 per second.

53. A method of generating mammalian embryos as described in claim 40, further comprising the step of freezing said semen.

54. A method of generating mammalian embryos as described in claims 51, further comprising the step of freezing said semen.

55. A method of generating mammalian embryos as described in claim 53, further comprising the step of thawing said semen.

56. A method of generating mammalian embryos as described in claim 54, further comprising the step of thawing said semen.

57. A method of producing a mammal having a predetermined sex comprising the steps of:

a. collecting semen from a male mammal;

b. freezing said semen;

c. thawing said semen;

b. determining the sex characteristic of a plurality of sperm cells contained within said frozen-thawed semen;

c. separating said sperm cells according to the determination of their sex characteristic;

d. isolating sperm cells separated according to the determination of their sex in a collection element;

d. establishing an artificial insemination sample from said sperm cells isolated in said collection element;

e. inserting said artificial insemination sample into a female mammal of the same species from which said semen was collected;

f. fertilizing at least one egg within said female mammal; and

g. producing an offspring mammal of the desired sex.

58. A method of producing a mammal having a predetermined sex as described in claim 57, wherein said male mammal is selected from the group of mammals consisting of primates, humans, swine, ovids, bovids, equids, canids, felids, and dolphins.

59. A method of producing a mammal having a predetermined sex as described in claim 58, further comprising the step of staining DNA within said sperm cells with a concentration of Hoechst 33342 greater than 40 micro-molar.

60. A method of producing a mammal having a predetermined sex as described in claim 59, wherein said step of staining DNA within said sperm cells with a concentration of Hoechst 33342 greater than 40 micro-molar comprises staining of sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85%.

61. A method of producing a mammal having a predetermined sex as described in claim 59, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is between about 200 micro-molar and about 2500 micro-molar.

62. A method of producing a mammal having a predetermined sex as described in claim 61, wherein said male mammal comprises said bovid, and wherein said concentration of Hoechst 33342 stain is 224 micro-molar.

63. A method of producing a mammal having a predetermined sex as described in claim 61, wherein said male mammal comprises said bovid and wherein said concentration of Hoechst 3342 stain is 2240 micro-molar.

64. A method of producing a mammal having a predetermined sex as described in claim 61, further comprising the step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes.

65. A method of producing a mammal having a predetermined sex as described in claim

62, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 190 minutes.

66. A method of producing a mammal having a predetermined sex as described in claim 63, wherein said step of adjusting a duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain between about 50 minutes and about 200 minutes comprises adjusting said duration of time said sperm cells are incubated with said concentration of Hoechst 33342 stain to about 60 minutes.

67. A method of producing a mammal having a predetermined sex as described in claims 57, 58, 61, 62, 63, 64, 65, or 66, wherein said step of staining DNA within said sperm cells with a concentration of Hoechst 33342 greater than 40 micro-molar comprises staining of sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises a rate selected from the group consisting of 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%.

68. A method of producing a mammal having a predetermined sex as described in claim 67, wherein said step of staining DNA within said sperm cells with sufficient uniformity to allow X-chromosome bearing sperm cells to be differentiated from Y-chromosome bearing sperm cells based upon the magnitude of fluorescence at a rate of greater than about 85% comprises differentiating said magnitude of fluorescence with a flow cytometer.

69. A method of staining sperm cells collected from mammals as described in claim 68, wherein said step of isolating sperm cells separated according to the determination of their sex in a collection element comprises isolating Y-chromosome bearing sperm cells into a separate collection element at a rate of about 1000 per second.

70. A method of staining sperm cells collected from mammals as described in claim 68, wherein said step of isolating sperm cells separated according to the determination of their sex in a collection element comprises isolating X-chromosome bearing sperm cells into a separate collection element at a rate of about 1000 per second.

71. A method of producing a mammal having a predetermined sex as described in claim 57, further comprising the step of limiting the number of isolated sperm cells in said artificial insemination sample to about 10% to about 50% of the number of said sperm cells relative to a typical unseparated artificial insemination sample.

72. A method of producing a mammal having a predetermined sex as described in claim 58, wherein said mammal comprises said bovid and wherein said artificial insemination sample has the number of isolated sperm cells limited to about one million to three million.

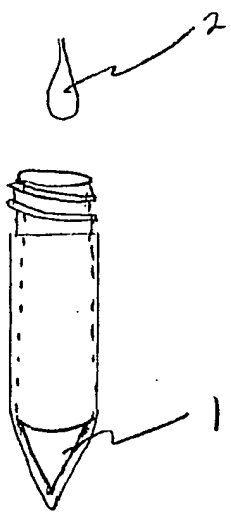
73. A method of producing a mammal having a predetermined sex as described in claim 58, wherein said mammal is a bovid and wherein said artificial insemination sample has the number of isolated sperm cells limited to between about one-hundred and fifty thousand and about one million.

74. A method of producing a mammal having a predetermined sex as described in claim 57, wherein said mammal comprises said equid and wherein said artificial insemination sample has the number of isolated sperm cells limited to between about forty million and about one hundred million.

75. A method of producing a mammal having a predetermined sex as described in claims 71, 72, 73 or 74, further comprising the step of creating superovulation in said female mammal to create at least two eggs comprising the step of using an ovulatory pharmaceutical to cause multiple eggs to be produced, and wherein said ovulatory pharmaceutical is injected in half day increments between any of days 2 and 18 of

the estrus cycle.

FIG 1



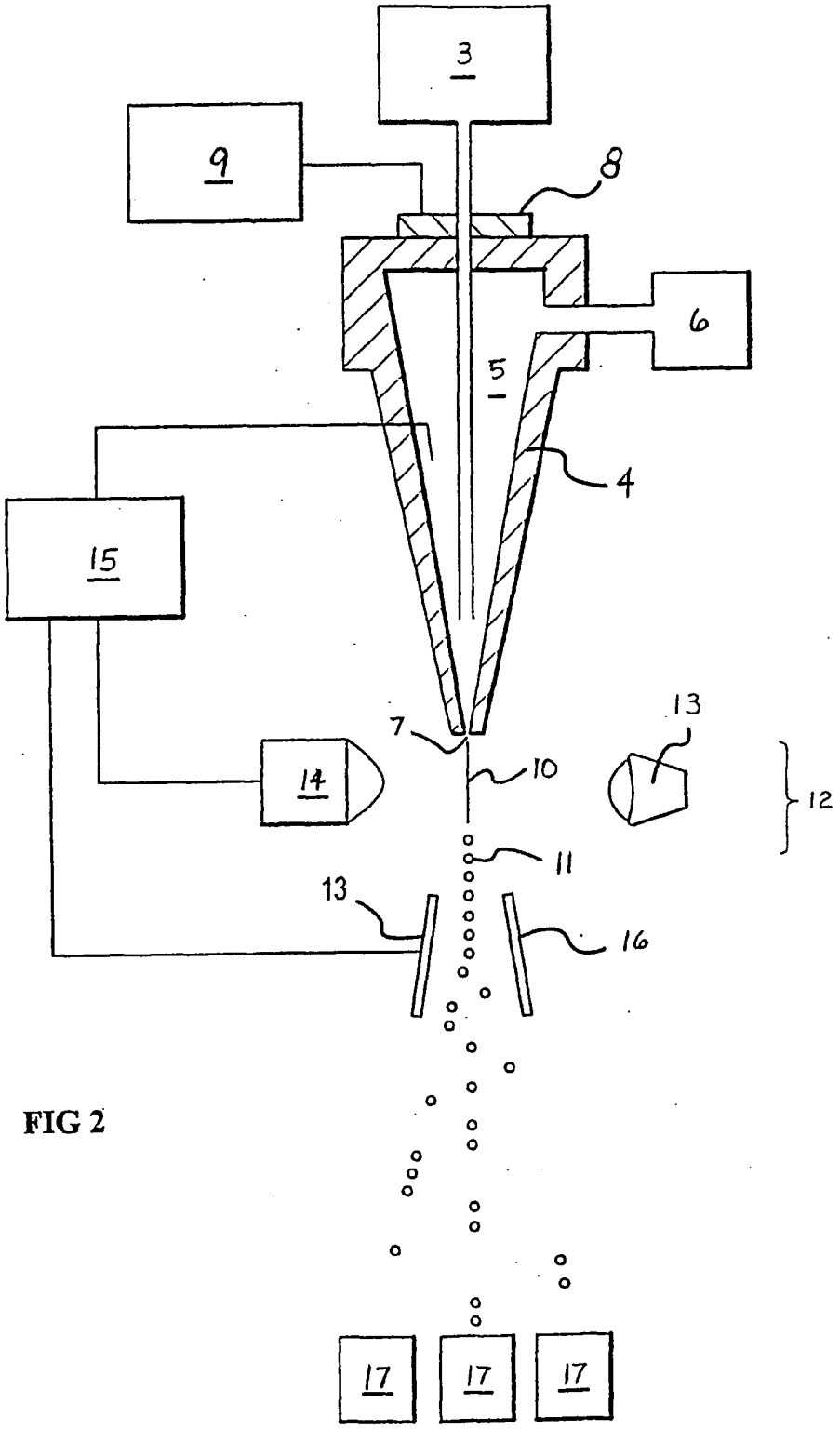


FIG 2

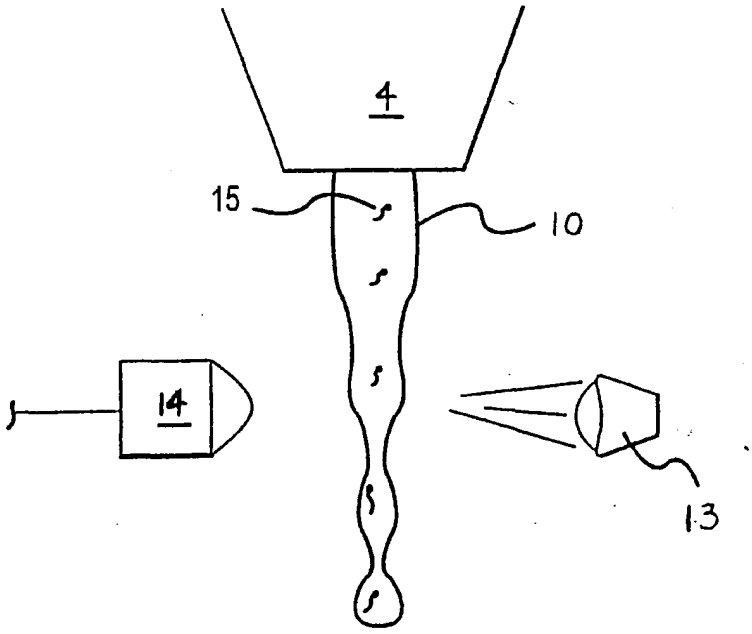


FIG 3